

ESSENTIAL GUIDES FOR ISOLATION/PURIFICATION OF CELLS

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In cell-separation technology the term 'component' of a mixture corresponds to a group of cells, which is usually called a cell population and shares a number of common features. How many common features a group of cells has to share in order to be called a cell population depends on the interest of the 'separator'. For example, a T-cell population may be a group of mononuclear white blood cells bearing CD3 antigens, while a helper cell population usually comprises mononuclear white blood cells bearing CD3 and CD4 antigens.

Cells metabolize as long as they live independently, whether they remain in an actual state of activation or differentiation or they proceed to another one. This means that a whole cell must not change its appearance or functions, but some cell components are chemically modified either anabolically or catabolically. So for discussing cell separation the term 'chemical modification' should be converted to 'biological modification' and in this chapter the expression 'without biological modification' will be defined as purification of cells without changes or signals for changes of cellular states of activation and/or differentiation.

No technology has been developed so far which allows picking of cell populations directly out of pieces of plant or animal tissues. So a 'mixture' which will be separated is normally a suspension of single cells prepared from parts of plants, from organs or body fluids of animals and humans or from two- or three-dimensional *in vitro* cell cultures. These cell sources already contain preselected groups of cells, the so-called organ or fluid (e.g. blood) specific cells. Still, a series of populations differing in important features are present in most plant or animal body compartments. In these instances, it may be of interest to separate cells for studying their biology or for using some of their capabilities in medicine or biotechnology.

Thus the following reflections on essential guides for separation/purification of cells are based on separations defined as processes of any scale by which cell populations of single-cell suspensions are separated from each other without biological modification.

Methods for Cell Separation without Biological Modification

The above definition rules out some technologies, frequently used to prepare homogeneous cell populations, because they include biological modifications of cells. For example, the enrichment of cell types of interest by establishing cell lines or cell clones will not be considered as a subject in this chapter. Cell lines or cell clones may be very useful sources of important genes and gene products. However, their cells are transformed in unnaturally fast growing states, in order to separate them from unwanted accompanying cells. Also cell separation/purification techniques using different capabilities of various kinds of cells to adhere to surfaces of, for example, culture dishes or fibres or to bind antibodies or macromolecules labelled by fluorescence dyes or magnetic beads, will not be described, because cell interactions with foreign components or antibody binding sites very often induce biological modifications. Of course, cell-purification methods like those mentioned above are very useful in research and biotechnology. The reader may find more informations regarding these techniques in the Further Reading.

This chapter focuses on application of counter-current centrifugal elutriation (CCE) and free-flow electrophoresis (FFE). These methods use differences of physical cell parameters such as specific cell density, cell size or negative surface charge density but do not include steps of cell labelling or cell transformation. They have the advantage that cells can be purified within a short time while they are kept suspended in biocompatible fluids or even culture media. Cell contacts to foreign surfaces and/or biologically active substances are thereby minimized and signals of activation and differentiation are delivered to cells during the isolation procedure to a minimal extent. Both methods may help to obtain sufficient numbers of identical cells with a high degree of purity and vitality for studying the biological role, which a defined cell population may play within an organism or for transplantation of cells with states of activation and differentiation suitable to fit in the new organism of a recipient.

Single-Cell Suspensions

Up to the present, cell separation by physical methods has required single-cell suspensions. Some cell

compartments such as peripheral blood, ascites, lymph or other body fluids already contain single cells. Cells of organs such as bone marrow, spleen or thymus can easily be removed, for example with the help of needles. The dissociation of single cells from two- or three-dimensional tissue cultures and from solid body tissues needs more rigorous methods. These cells not only adhere to each other, but are also more or less firmly attached to the extracellular matrix, a complex network of collagen, proteoglycans and cross-linking proteins such as laminin and fibronectin. Mechanical dissociation by scraping cell monolayers from their surfaces or by forcing tissue pieces or cell aggregates through orifices or syringes or pipettes very often damages the cells and results in a poor yield. An enzymatic treatment or pretreatment of cell cultures or organs is thus often applied in order to digest the extracellular matrix and/or to weaken the cell-cell attachment sites. The selection of the enzymes, their concentration and their time and temperature of application depend on the type of organ and its originating organism. Enzymes frequently used for animal cell preparation are collagenase, trypsin, pronase, dispase, papain, chymotrypsin, hyaluronidase, lysozyme and DNase, while cellulase is a typical enzyme for plant tissue dissociation. Sometimes the action of the enzymes is supported by the presence of EDTA (ethylenediaminetetraacetic acid), which destroys binding sites mediated by Ca^{2+} ions. Details regarding techniques of preparing single cells may be found in books quoted in the Further Reading. In general, the enzymatic treatment has to be optimized for each cell-separation process, because the enzymes may not only attack cell membrane components which keep the cells within the tissue but may also destroy important cell-membrane functions.

If neither mechanical nor enzymatic methods lead to satisfactory results, an alternative way may be to incubate pieces of tissue on surfaces which challenge the cells to move out of the tissue and to form monolayers surrounding the tissue. For example, cells of human prostate tumour sections, which cannot be dissociated in viable single cells by a number of mechanical and enzymatic techniques, migrate out of the tissue and form a monolayer, when incubated in culture dishes for a few weeks. After removal of the tissue, the cells can easily be scraped off the plastic dish surface.

Pre-Separation

As soon as single cells are available, countercurrent centrifugal elutriation or cell electrophoresis may be applied. However, some cell-separation tasks need pre-enrichment of the cells of interest. Especially

where an investigator is interested in a peripheral blood leukocyte population such as a lymphocyte, granulocyte, monocyte or even reticulocyte population, the erythrocytes comprising more than 99% of the blood cells have to be removed before one of these white blood cell populations may be purified. In these instances, it has proved useful to perform a first step of density-gradient centrifugation, which does not need pre-labelling of cells. The method allows the separation of mononuclear leukocytes consisting mainly of lymphocytes and monocytes from granulocytes and erythrocytes. The separation principle is based on different specific densities of the various cell populations. In practice, a tube is filled with a biocompatible isotonic medium with a specific density adjusted between the specific densities of the cells to be separated and the cell mixture is layered on the top of this medium. Then the whole sample is exposed to a few hundred *g* by centrifugation. The forces cause mononuclear leukocytes with a density lower than the separation medium to remain at the top, while those with higher density sediment to the bottom. The specific density of the medium is adjusted by silica colloids, which are coated with an inert material and have low osmolality. Although modern commercially available density-gradient separation media are very inert and direct damage of the cells is seldom observed, the silica colloids are pinocytosed by some cells.

If this is a problem, prolonged centrifugation of whole blood may be an alternative route. During such a centrifugation procedure, a layer of white blood cells is formed above the erythrocytes. This layer, called a buffy coat, contains mononuclear as well as polymorphonuclear leukocytes and lies directly on the erythrocytes. The white cells may be collected. Although co-collection of a considerable number of red cells is usually unavoidable, a degree of white blood cell pre-enrichment can be achieved which allows reasonable further separation by, e.g. CCE.

Countercurrent Centrifugal Elutriation (CCE)

The method of CCE and the equipment required for cell separation according to cell size have already been described so they are summarized only brief here. Cells loaded into the elutriation chamber are subjected to centrifugal sedimentation forces generated by rotation in an outward direction and to counterflow fluid forces pumped into the separation chamber in an inward direction. As long as sedimentation forces are balanced by the opposite fluid forces, different cell populations take different chamber positions depending mainly on their sizes



Figure 1 Table-top elutriator (middle) together with an infusion pump driving the counterflow (left) and a volume analyser (right).

and to a lesser extent on their specific densities. If the counterflow rate is increased by speeding up a pump and/or the sedimentation forces are decreased by reducing centrifugation velocity, the various cell populations are washed out sequentially with increasing size ranges.

Commercially, small elutriation chambers with 5 mL separation volume and large ones with 40 mL are available together with suitable centrifuges and rotors from Beckman Instruments (Palo Alto, USA). They can accommodate up to 10^9 and 10^{10} cells, respectively. A laboratory device has also been constructed; it consists of a table-top centrifuge with a small rotor which has a separation chamber with a volume of 0.5 mL to which 10^6 tissue cells or 2×10^7 mononuclear leukocytes may be loaded (Figure 1).

In order to fractionate cells with different sizes into different fractions, counterflow rates and rotor speeds have to be adjusted depending on rotor size, chamber volume and the size of the cells to be separated. The result of each separation should be controlled by recording volume distributions of the cells of each fraction with the help of cell size analysers. Beckman

rotors are frequently operated at speeds ranging from 1000 to 4000 rpm. Dependent on the actual rotor speed, the counterflow through small chambers may be started at rates between 8 and 20 mL min⁻¹ and increased for fractional elution stepwise up to 100 mL min⁻¹ (Table 1); counterflow rates through large separation chambers may start at 50 mL min⁻¹. The table-top centrifuge is operated at counterflow rates between 1 and 6 mL min⁻¹, while the rotation speeds are between 500 and 2200 rpm for tissue cell separation and between 1500 and 2800 rpm for leukocyte separation (Table 2). Using any of the instruments, separation times are short and the cells may be kept suspended in culture medium. Thus unwanted exposure of the cells to stimulatory environments are minimized so that characteristics of separated populations will rather closely reflect the status of the original cells before fractionation.

Because of these advantages, CCE has proved most useful, if applied for the separation tasks listed below:

- For cell cycle analyses, the cellular DNA content is normally determined. However, cells have to be killed in order to make their DNA accessible for intercalating fluorescence molecules. If living cells in different steps of the cell cycle need to be separated, an increase of cell size during passage through the cell cycle may be used as a separation parameter. With the help of CCE the small cells, which are in G1 phase can be separated from S-phase cells which have intermediary size and from the G2/M-phase cells which have the largest size of the cell population. Many flow cytometric analyses of the DNA content of separated cells have already proved that CCE enables the separation of cells of cell lines in fractions, which have up to 100% G1 phase cells, up to 80% S-phase cells and up to 80% G2/M phase cells, respectively.
- A number of different kinds of cells such as mononuclear phagocytes recognize very non-specifically

Table 1 Examples of counterflow and rotor speed adjustments using a Beckman elutriator equipped with JE-6 rotor (small separation chamber)

Cell mixtures:	sheep erythrocytes/reticulocytes	human mononuclear leukocytes	cultured human lymphocytes/macrophages
Pre-enrichment:	buffy coat	density-gradient centrifugation	none
Cell size range:	28–42 μm^3	180–400 μm^3	180–2000 μm^3
Rotor speed:	3000 rpm	2460 rpm	2460 rpm
Counterflow:	9–24 mL min ⁻¹	16.5–40 mL min ⁻¹	16.5–81 mL min ⁻¹
Desired cells:	reticulocytes	lymphocytes/monocytes	macrophages
Eluted at:	24 mL min ⁻¹	22 mL min ⁻¹ /40 mL min ⁻¹	80 mL min ⁻¹
Use:	analysis of volume regulation	further enrichment, immunological tests	surface charge analysis

For details, see: Lauf PK and Bauer J (1987) *Biochemical and Biophysical Research Communications* 144: 849–855 and Bauer J and Hannig K (1984) *Electrophoresis* 5: 269–274.

Table 2 Examples of counterflow and rotor speed adjustments using the self-made table-top elutriator

Cell mixture:	cultured human mononuclear leukocytes	human erythrocytes/granulocytes	cultured human tissue cells
Pre-enrichment:	none	buffy coat	none
Cell size range:	180–2000 μm^3	90–400 μm^3	1000–3000 μm^3
Rotor speed:	2800, 500 rpm	2800, 1500 rpm	2200–500 rpm
Counterflow:	2.5, 4, 6 mL min^{-1}	4–6 mL min^{-1}	4–6 mL min^{-1}
Desired cells:	antibody-producing cells	granulocytes	hyperdiploid cells
Eluted at:	6 mL min^{-1} /500 rpm	6 mL min^{-1} /1500 rpm	6 mL min^{-1} /500 rpm
Use:	antibody secretion	analysis	analysis

For details, see: Bauer J and Hannig K (1988) *Journal of Immunological Methods* 112: 213–218 and Bauer J, Grimm D, Hofstaedter F and Wieland W (1992) *Biotechnological Progress* 8: 494–500.

foreign molecules and particles entering an organism. So despite many alternative methods such as antibody-dependent sorting or panning, CCE, which does not involve cell adhesion to matrices or to antibodies, is often preferred, to separate monocytes from peripheral blood or bone marrow and to purify macrophages from alveolar tissues or Kupffer cells from liver and to enrich mast cells, if contacts to stimulatory surfaces and substances must be avoided.

- Problems still exist in the detailed study of the biological and physiological features of healthy and malignant animal tissue cells and plant protoplasts. These cells have not yet been characterized, as well as, for example, lymphoid cells. Antibodies against the surface epitopes of such cells are not isolated in great abundance, so fractionation of single-cell populations, obtained from tissues of various organisms, by CCE, is a competitive way to provide important homogeneous cell populations for biological, toxicological and pharmacological studies.
- CD34-positive hematopoietic stem cells are very helpful to restore hematopoiesis of patients, who have to undergo whole-body radiation or rigorous chemotherapy. In the past, CD34-positive cells were separated either by panning, immunomagnetic sorting or fluorescence-activated cell sorting. All these techniques include expensive time-consuming steps of labelling cells by antibodies and generate problems of removing the antibodies/ligands from the surface of the purified cells. CCE thus appears to be an alternative method for CD34-positive stem cell purification as the stem cells have a similar volume as mononuclear leukocytes. However, resolution improvements still seem to be necessary.

Free-Flow Cell Electrophoresis (FFE)

Another method for purifying cell populations without antibody tagging or cell adherence is free-flow

electrophoresis. Its basic principle has already been described and is repeated briefly here. A laminar buffer stream flows between two narrowly spaced parallel glass plates forming a separation chamber. Near one end of the chamber, a cell suspension is injected as a narrow band into the fluid flow which carries the cells through an electric field applied perpendicularly to the carrier fluid flow. Cells exposed to the electric field migrate laterally towards the positively charged electrode with velocities depending on their negative surface charge densities. Thus cells with different negative surface charge densities migrate at different speeds, arrive at different points along the opposite edge line and can be collected for preparative isolation.

This principle is called ‘free-flow zone electrophoresis’ (FFZE) and is still the only electrophoresis mode applicable to cell separation, although it has poorer resolution than other electrophoresis modes such as isoelectric focusing (IEF) and isotachopheresis (ITP), because it is a non-focusing process. In addition, most whole cells do not tolerate a fluid pH below 6.9 and above 7.5 and need media which allow reasonable electrophoretic mobilities, but are simultaneously biocompatible. So for quite a long time, cell electrophoresis was rarely applied, particularly as resolution was often not high enough to purify cell populations with different mean electrophoretic mobilities but overlapping distribution curves and this second drawback negatively influenced cell vitality. Cells had to be suspended in media lacking NaCl or other physiologically important ions, because too many ions in the chamber medium caused problems of performance such as overheating of the medium and short electromigration distances, as long as only conventional devices with homogeneous chamber media were available.

Recently, a new type of FFE was developed which opened new possibilities of electrophoretic cell separation. It is called Octopus and is commercially available from the Dr. Weber GmbH, Kirchheim, Germany (Figure 2). It is quite suitable to perform

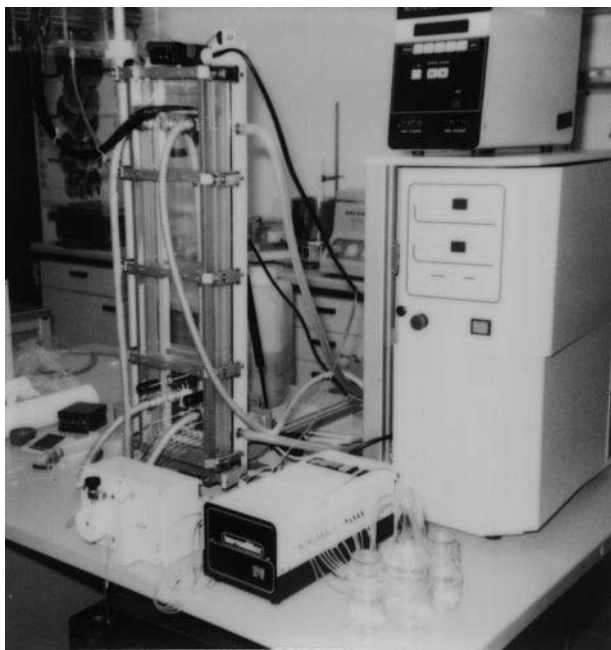


Figure 2 An Octopus free-flow electrophoresis apparatus with the electrophoresis chamber in vertical position (left) and implements such as a power supply, a pump and the control unit (right). (A generous gift of the Dr. Weber GmbH, D-85551 Kirchheim, Germany. More information about the machine may be found at <http://members.aol.com/ffeweber/default.htm>.)

preparative cell electrophoresis but can easily be adjusted to IEF and ITP of sub-cellular particles or molecular substances. Its electrophoresis chamber has a length of 500 mm and a width of 100 mm and can be fixed in a vertical or a horizontal position, as long as specimen sedimentation does not play a role. The thickness is variable, between 0.4 mm and 0.2 mm, so that heat-removal efficiency may be enhanced, if ions are required in cell suspension media and the application of high electric fields is necessary. An optical particle detection system allows control of process stability.

The major advantage of the new system is that various media may flow through the chamber adjacent to each other and the sample may be introduced at the optimal site (Figure 3). This means for cell electrophoresis, that one central cell suspension medium, which may contain up to 50 mmol L^{-1} NaCl is pumped between two margin media with elevated quantities of ions flowing at both edges (Table 3). They cover the electrode membranes, protect the separation medium from detrimental influences of the electrodes, prevent diminution of Na^+ and Cl^- ion concentrations within the central chamber area and conduct the electric current to this area of cell transport with minimal voltage drop.

Like CCE, FFE is most advantageous if antibodies coupled to fluorescent dyes or magnetic beads are not

available or must not be applied. So the method is quite useful, when cells are separated by CCE because of the reasons explained above and the resulting fractions still contain cells which belong to different populations, but have equal size, while their electrophoretic mobilities are different. For example, cell fractions are routinely obtained, which contain more than 90% monocytes, if pre-enriched mononuclear leukocytes are elutriated. In such fractions, up to 0.2% antibody-producing cells with equal size as monocytes but different electrophoretic mobilities (EPM) are often co-collected. The antibody-producing cells can be further enriched by FFE. Similarly, T-cell fractions obtained by CCE contaminated by accessory cells, of equal size have been submitted to a following step of FFE purification. T-cells of individual blood donors were obtained, which did not respond to concanavalin A unless accessory cells were re-added.

A cell feature, which cannot be defined by antibodies is the negative surface charge density. Its biological role is still very poorly understood. Observations made during recent cell electrophoretic studies appear currently like very scattered mosaic stones which do not allow the whole picture to be revealed. For example, erythrocytes change their EPM in patients suffering various kinds of diseases, monocytes change their EPM when maturing to non-activated macrophages, B-cells change their EPM when developing to antibody-producing cells *in vivo* but not *in vitro*, and mice with different erythrocyte EPM have different sensitivities to malaria infection (see Further Reading). These accumulating data suggest that further efforts in studying the biological relevance of the negative surface charge density by FFE will be worthwhile.

Since electrophoresis media with $20\text{--}50 \text{ mmol L}^{-1}$ NaCl can be used for cell separation, tissue cells can be processed without clotting. Now it is possible to electrophorese cell suspensions obtained from tissues directly or indirectly after a few passages of culture. The separations performed so far have revealed quite interesting new tissue cell sub-populations. Hence, future application of FFE to fractionation of viable tissue cells appears promising.

Conclusions

Essential guides for separation/purification of cells have been described in this chapter following a definition of cell separations as processes of any scale by which cell populations are separated from each other without biological modification, i.e. without changes of their actual states of activation and differentiation. As explained above and shown in Figure 4 single cells

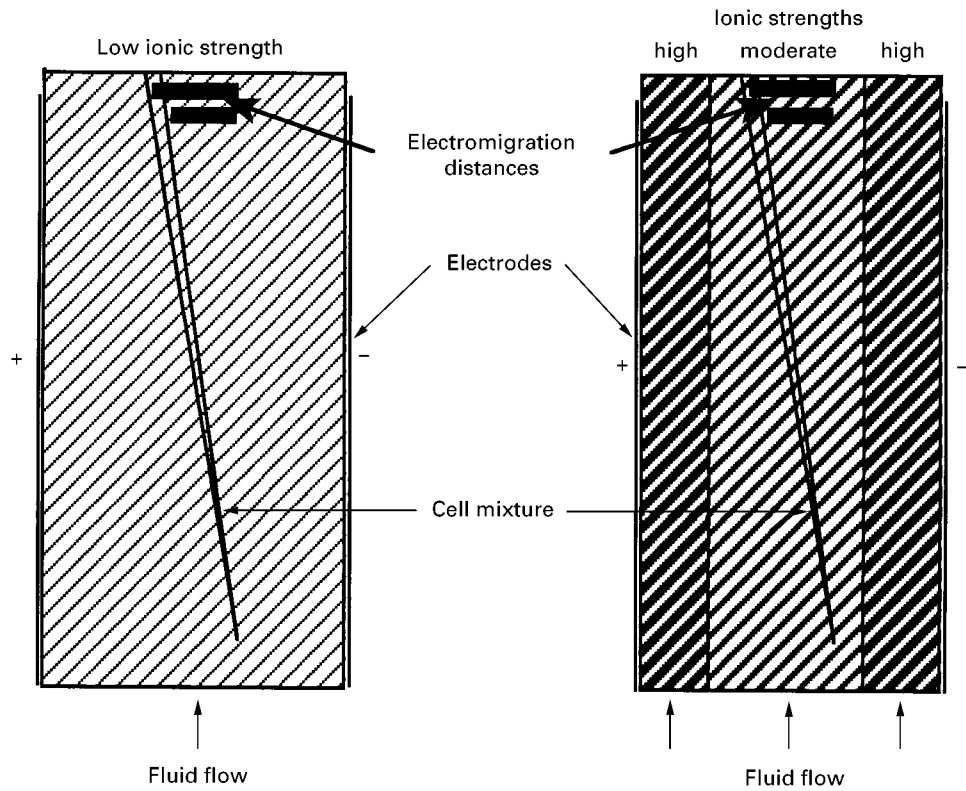


Figure 3 Scheme of free flow electrophoresis chambers working with homogeneous (left) and segmented (right) carrier fluids.

suspended in suitable media after preparation from human or animal body fluids, from human, animal or plant tissues or from *in vitro* cultures are prerequisites of such processes. If a cell suspension with a reasonable number of desired cells is available, methods such as countercurrent centrifugal elutriation and free-flow electrophoresis may be applied, either each

of them alone or in combination. As both cell-separation methods are rapid and work while cells are kept in suspensions with minimal contact with foreign surfaces but do not require labelling of cell surfaces by antibodies or other macromolecules, a fair chance can be expected to obtain homogeneous cell populations retaining their original states of activation and

Table 3 Examples of buffer systems for homogeneous and segmented FFE chamber fluids

Cell-suspension medium	Margin buffers	Electrode buffer(s)
<i>Homogeneous</i>		
27 mmol L ⁻¹ triethanolamine 4 mmol L ⁻¹ potassium acetate 27 mmol L ⁻¹ sucrose 1 mmol L ⁻¹ glucose 216 mmol L ⁻¹ glycine pH 7.2 adjusted by acetic acid		342 mmol L ⁻¹ triethanolamine 40 mmol L ⁻¹ potassium acetate pH 7.2 adjusted by acetic acid
<i>Segmented</i>		
central: 10 mmol L ⁻¹ triethanolamine 2 mmol L ⁻¹ sodium acetate 50 mmol L ⁻¹ NaCl 2 mmol L ⁻¹ glucose 180 mmol L ⁻¹ sucrose pH 7.2 adjusted by acetic acid	anodal: 50 mmol L ⁻¹ triethanolamine 250 mmol L ⁻¹ Na ₂ SO ₃ pH 7.2 adjusted by acetic acid cathodal: 50 mmol L ⁻¹ triethanolamine 250 mmol L ⁻¹ NaCl 75 mmol L ⁻¹ sucrose pH 7.2 adjusted by acetic acid	anodal: 200 mmol L ⁻¹ sodium acetate cathodal: 100 mmol L ⁻¹ HCl 100 mmol L ⁻¹ NaCl 200 mmol L ⁻¹ imidazole

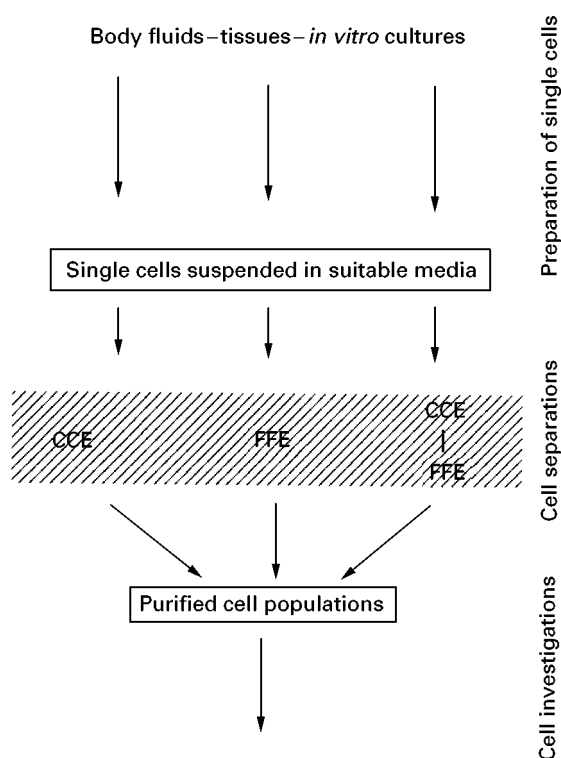


Figure 4 Flow diagram showing a survey of the processes of cell purification described in this article.

differentiation, even if appropriate antibodies are not available.

Cells purified without biological modifications may be especially useful if it is of interest to study their original *in vivo* status or to use them for transplantation purposes and if size or surface charge-related phenomena are to be investigated. As knowledge of possible cellular characteristics and components is continuously accumulating, questions on their actual

expression under normal and pathological conditions will frequently arise. For studying such questions, homogeneous cell populations retaining their original *in vivo* status may become so important that techniques and instruments required for their purification will be further improved.

See also: **Cells and Cell Organelles: Field Flow Fractionation.**

Further Reading

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ESSENTIAL GUIDES FOR ISOLATION/PURIFICATION OF DRUG METABOLITES

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Metabolite Isolation and Identification

Following the administration of drugs to either animals or man, very few of the drugs are excreted

unchanged. The majority undergo biotransformations by interaction with a complex series of enzymes. This process, known as drug metabolism, is not restricted to drugs but occurs with all chemicals that are taken in by living systems, including food additives, pesticides, carcinogens, etc. These chemicals are termed exogenous compounds, as opposed to endogenous, or naturally present, compounds.

Metabolic studies have made, and continue to make, fundamental contributions to the drug